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# Dietary fish oil delays hypoxic skeletal muscle fatigue and enhances caffeine-stimulated contractile recovery in the rat in vivo hindlimb

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Oxygen efficiency influences skeletal muscle contractile function during physiological hypoxia. Dietary fish oil, providing docosahexaenoic acid (DHA), reduces the oxygen cost of muscle contraction. This study used autologous perfused rat hindlimb model to examine the effects of a fish oil diet on skeletal muscle fatigue during an acute hypoxic challenge. Male Wistar rats were fed a diet rich in saturated fat (SF), long chain (LC) n-6 polyunsaturated fatty acids (n-6 PUFA), or LC n-3 PUFA DHA from fish oil (FO) (8weeks). During anaesthetised and ventilated conditions (normoxia 21% O2 [SaO2-98%] and hypoxia 14% O2 [SaO2-89%]) the hindlimb was perfused at a constant flow and the gastrocnemius-plantaris-soleus muscle bundle was stimulated via sciatic nerve (2Hz, 6-12V, 0.05ms) to established fatigue. Caffeine (2.5, 5, 10mM) was supplied to the contracting muscle bundle via the arterial cannula to assess force recovery. Hypoxia, independent of diet, attenuated maximal twitch tension (normoxia: 82±8; hypoxia 41±2g.g-1 tissue w.w.). However, rats fed fish oil sustained higher peak twitch tension compared to the SF and n-6 PUFA groups ($P<0.05$) and the time to decline to 50% of maximum twitch tension was extended (SF; 546±58, n-6PUFA; 522±58, FO; 792±96 s; $P<0.05$). In addition, caffeine stimulated skeletal muscle contractile recovery was enhanced in the fish oil fed animals (SF; 41±3, n-6PUFA; 40±4, FO; 52±7% recovery; $P<0.05$). These results support a physiological role of DHA in skeletal muscle membranes when exposed to low-oxygen stress that is consistent with the attenuation of muscle fatigue under physiologically normoxic conditions.

**Keywords**

delays, hypoxic, skeletal, muscle, fatigue, enhances, contractile, oil, recovery, dietary, rat, vivo, hindlimb, fish, caffeine-stimulated

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**Running title:** Dietary fish oil and hypoxic muscle fatigue

## Abstract

Oxygen efficiency influences skeletal muscle contractile function during physiological hypoxia. Dietary fish oil, providing docosahexaenoic acid (DHA), reduces the oxygen cost of muscle contraction. This study used autologous perfused rat hindlimb model to examine the effects of a fish oil diet on skeletal muscle fatigue during an acute hypoxic challenge. Male Wistar rats were fed a diet rich in saturated fat (SF), long chain (LC) n-6 polyunsaturated fatty acids (n-6 PUFA), or LC n-3 PUFA DHA from fish oil (FO) (8weeks). During anaesthetised and ventilated conditions (normoxia 21% $O_2$ [$SaO_2$-98%] and hypoxia 14% $O_2$ [$SaO_2$-89%]) the hindlimb was perfused at a constant flow and the gastrocnemius-plantaris-soleus muscle bundle was stimulated via sciatic nerve (2Hz, 6-12V, 0.05ms) to established fatigue. Caffeine (2.5, 5, 10mM) was supplied to the contracting muscle bundle via the arterial cannula to assess force recovery. Hypoxia, independent of diet, attenuated maximal twitch tension (normoxia: 82±8; hypoxia 41±2g.g$^{-1}$ tissue w.w.). However, rats fed fish oil sustained higher peak twitch tension compared to the SF and n-6 PUFA groups ($P<0.05$) and the time to decline to 50% of maximum twitch tension was extended (SF; 546±58, n-6PUFA; 522±58, FO; 792±96 s; $P<0.05$). In addition, caffeine stimulated skeletal muscle contractile recovery was enhanced in the fish oil fed animals (SF; 41±3, n-6PUFA; 40±4, FO; 52±7% recovery; $P<0.05$). These results support a physiological role of DHA in skeletal muscle membranes when exposed to low-oxygen stress that is consistent with the attenuation of muscle fatigue under physiologically normoxic conditions.


**Key Words:** fish oil, LC n-3 PUFA, docosahexaenoic acid, muscle fatigue, hypoxia.

## Introduction

Exercise performance is compromised during an hypoxic challenge (Gore *et al.* 1997) whereby a reduction in oxygen supply contributes to physiological alterations in cardiovascular and respiratory function (Peltonen *et al.* 2001). Skeletal muscle contractile force is also reduced and partly independent of the low oxygen delivery *per se* (Perrey *et al.* 2009), which suggests non-haematological mechanisms, including improved exercise economy (Gore *et al.* 2007), as new approaches to improve physiological function in low oxygen environments.

When fish oil is provided in the diet, the concentration of the long-chain omega-3 polyunsaturated fatty acid (LCn-3PUFA) docosahexaenoic acid (DHA) increases proportionately in cellular membranes of muscle (Andersson *et al.* 2002; Henry *et al.* 2015) as in other organs (Charnock *et al.* 1992). The majority of research into effects of LCn-3PUFA on striated muscle physiology has focussed on myocardium. Notwithstanding the physiological differences between cardiac and skeletal muscle, the observation that myocardial oxygen consumption is lower for any corresponding increase in external work during increasing preload (Pepe *et al.* 2002; McLennan *et al.* 2012) is directly relevant. These efficiency improvements, together with the well-established prevention of ischaemia- and reperfusion-induced cardiac arrhythmias, are at least partly attributable to attenuated cellular $Ca^{2+}$ overload (Pepe and McLennan 2002;McLennan 2014). Furthermore, post-ischaemic cardiac contractile recovery is improved following fish oil feeding (Pepe *et al.* 2002; Abdukeyum *et al.* 2008) highlighting the physiological role of membrane incorporated DHA in contractile function when oxygen supply is compromised.

Skeletal muscles share with myocardium, a unique capacity to incorporate membrane DHA from the diet, well above circulating levels (Charnock, Abeywardena *et al.* 1992; Henry *et al.* 2015) with a suggestion that it may be essential for optimal physiological function. All striated muscle is regulated according to oxygen supply and demand (contractile force). We have previously demonstrated that dietary fish oil delays skeletal muscle fatigue during normoxic conditions (Peoples *et al.* 2014) and improves contractile recovery (Peoples *et al.* 2010) whereby modulation of oxygen cost and sustained contractions are associated with muscles dominated by a relative high energy cost (Henry, *et al.* 2015). Importantly, DHA appears to have physiological roles other than competing with or replacing the plant based n-6 family of PUFA that predominate in the western diet (McLennan 2014). This includes nutritional preconditioning through upregulation of anti-oxidant systems that has been likened to the more widely recognised ischaemic (Abdukeyum *et al.* 2015) or repeated ischemic preconditioning (Thijssen *et al.,* 2016). Thus, our first objective, using the autologous pump-perfused *in vivo* rat hindlimb (Peoples *et al.* 2013), was to determine if the provision of a DHA-rich fish oil could attenuate contractile fatigue induced during an acute hypoxic challenge.

Skeletal muscle contraction, like the heart, is dependent on $Ca^{2+}$ handling (Allen *et al.* 1989; Westerblad *et al.* 1991). Hypoxic stress impairs skeletal muscle contraction (Brotto *et al.* 2000) whereby $Ca^{2+}$-activated force is further disrupted (Ottenheijm *et al.* 2006), likely as a response to avoid 'metabolic catastrophe' (MacIntosh *et al.* 2012). In contracting skeletal muscle, sarcoplasmic reticulum $Ca^{2+}$ release can be augmented by caffeine (Weber and Herz 1968), which can facilitate the return of muscle contractile force in a fatigued state in animals (Howlett *et al.* 2005) and in humans subjected to conditions of fatigue (Tarnopolsky and Cupido 2000). Caffeine has garnered popularity as an ergogenic aid (Graham 2001;

Burke 2008) and represents an ideal approach to investigate post-hypoxic contractile recovery. Thus our second objective was to determine if dietary fish oil could influence the caffeine-facilitated contractile recovery of the rat hindlimb.

The overall aim of this study was to investigate the role dietary fish oil, delivering long chain (LC) n-3 PUFA DHA, during conditions of skeletal muscle fatigue when oxygen supply is compromised. Furthermore, this study aimed to identify if contractile force recovery, using a caffeine stimulus, was enhanced following provision of fish oil. We hypothesised that hypoxia would exacerbate contractile fatigue and that a DHA-rich fish oil would attenuate this fatigue and augment caffeine-induced recovery.



## Materials and Methods

*Animals*

Eighteen young male Wistar rats (8-10weeks of age, initial body mass range 250-300g) were used for the study. Experiments were approved by the Animal Care and Ethics Committee from the University of Wollongong, and all national and institutional guidelines were followed. Animals were housed two per cage in the institution's animal facility with a room temperature maintained at 23$^{o}$C-25$^{o}$C and a 12 hour light-dark cycle.

*Diet composition*

Three diets (saturated fat (SF); n-6 PUFA; LC n-3 PUFA (FO)) were prepared for the current study as previously described (Peoples and McLennan 2010). In brief, the diets contained a balanced mix of macro and micronutrients based on the AIN 93M diet (Reeves *et al.* 1993), to avoid any nutritional deficiencies. The diets, all containing 10% fat by weight

were prepared from purified ingredients and stored at –20°C (Owen et al. 2004). If compared to a regular chow control, any effects of a fish oil diet could be attributed to: total PUFA content; presence of n-3 PUFA; or absence of n-6 PUFA. For this, as in previous studies, the proportions of fat sources (SF diet: 30:70 olive oil and beef tallow; n-6 PUFA diet: 50:50 olive oil and safflower oil; FO diet: 30:70 olive oil and tuna fish oil) were designed to deliver: i) similar total PUFA in the n-6 PUFA (36.65g.kg$^{-1}$) and FO (31.40g.kg$^{-1}$) diets; ii) similar (low) total n-6 PUFA in the FO (5.01g.kg$^{-1}$) and SF (4.77g.kg$^{-1}$) diets; and iii) similar (low) LC n-3 PUFA in the n-6 PUFA (0.49g.kg$^{-1}$) and SF (0.58g.kg$^{-1}$) diets (Peoples and McLennan 2010). Olive oil was provided as light (refined) oil and therefore a rich source of monounsaturated fatty acids free of confounding sources of natural antioxidants; saturated fat as beef tallow; n-6 PUFA as safflower oil; LC n-3 PUFA as high DHA tuna fish oil (α-linolenic acid 18:3 n-3 (ALA) 0.56g.kg$^{-1}$; EPA 4.87g.kg$^{-1}$; DHA 20.2g.kg$^{-1}$) (Nu-Mega Ingredients, Altona North, VIC, Australia).

*Study Design*

Animals were fed a fully fabricated olive oil diet for 14 days to wash out any potential contribution to muscle membrane fatty acid composition from LCn-3PUFA that may be in a chow diet, which often include fish meal as a primary protein source. The 18 rats (initial body mass range 250-300g on arrival) were then randomly allocated into three groups (n=6 per group) and fed the SF diet for 8 weeks (*ad libitum)*. Fresh food was provided twice per week and the daily consumption was estimated by weighing the remainder from the previous feeding.

153 *Surgical preparation for ventilation, muscle perfusion and stimulation.*

154 The rat *in vivo* autologous pump-perfused contracting hindlimb preparation has been
155 previously described (Peoples, *et al.* 2013). In brief, rats (20 weeks of age) were
156 anaesthetised (sodium pentobarbital 6mg.100g$^{-1}$ i.p., plus supplementary anaesthetic
157 (2mg.100g$^{-1}$ i.p.) as required) with body temperature maintained at 37°C (rectal temperature)
158 throughout. The trachea was cannulated for artificial ventilation (1mL.100g$^{-1}$ body weight;
159 Rodent Ventilator 7025, Ugo Basile, Italy). Systemic blood pressure was monitored via the
160 left carotid artery.

161

162 All cannulae, including extracorporeal pump circuit were fluid filled with saline containing
163 6% dextran (w/v) (Dextran 70, Sigma-Aldrich, Sydney, Australia) and 5000 IU
164 heparin.100ml$^{-1}$ (Sigma-Aldrich, NSW, Australia). Left femoral artery was perfused with
165 arterial (oxygenated) blood taken from the right femoral artery (non-perfused leg) (peristaltic
166 roller pump: Minipuls 3, Gilson, Middleton, WI), delivering blood directly to muscle groups
167 below the knee. Perfusion pressure was measured (pressure transducer: Argon CDXIII,
168 Maxim Medical, U.S.A.) distal to the pump. Hindlimb venous (de-oxygenated) blood was
169 returned to the heart and lungs via passive flow from the left femoral vein into the right
170 jugular vein to permit access for venous sampling.

171

172 Hindlimb muscles were stimulated to contract by sciatic nerve stimulation (bipolar electrode:
173 F-B5EI; Grass Technologies, West Warwick, RI) with the proximal nerve crushed to prevent
174 retrograde conduction. The gastrocnemius-plantaris-soleus muscle group tendons were tied
175 with non-compliant silk and connected to a force transducer (FT03C, Grass Technologies,
176 West Warwick,, RI). Saline-soaked gauze was placed over the muscles to prevent drying.

177

178 *Normoxic and hypoxic protocols*

179 During normoxic conditions the ventilator drew air from the room at sea level conditions
180 (21% $O_2$ and 0.03% $CO_2$). To create hypoxia and compromise oxygen delivery to the
181 contracting muscle, the external valve of the ventilator was connected to an airtight sampling
182 bag filled with a gas mixture containing low oxygen (14%), elevated carbon dioxide (~0.5%)
183 and remainder nitrogen (~85%). The sampling bag volume was large enough to sustain
184 animal ventilation for 15 minute periods and was re-filled as required during the experiment.
185
186 During normoxic conditions the hindlimb was perfused for 30 min at 1mL.min$^{-1}$ without
187 stimulation to allow perfusion pressure to reach steady state (~100mmHg). Arterial and
188 venous blood samples were drawn for calculation of resting oxygen consumption. Flow rate
189 was gradually increased to 2mL.min$^{-1}$ over three minutes, then the muscle was stimulated via
190 the sciatic nerve (2Hz, 7-12V, 0.05ms) to contract for three minutes. The stimulation duration
191 was sufficient to achieve maximum peak twitch tension but was limited to avoid muscle
192 fatigue prior to the 30 min of stimulation to follow. No blood was drawn during the normoxic
193 stimulation in order to save red blood cells and oxygen carrying capacity for the later
194 contraction period. After three minutes of normoxic contraction, perfusion flow rate was
195 returned to 1 mL.min$^{-1}$ for a 20 minutes recovery period.
196
197 Hypoxic conditions were commenced (ventilated with 14% oxygen) with the himdlimb flow
198 rate maintained at 1 mL.min$^{-1}$ for 5 min and resting baseline arterial and venous blood
199 samples were drawn for calculation of resting oxygen consumption under hypoxic conditions.
200 Hindlimb blood flow was then increased to 2 ml.min$^{-1}$ over three minutes and the 30 min
201 repetitive twitch stimulation bout (2Hz, 7-12V, 0.05ms) commenced. Venous samples were

collected throughout the stimulation bout at time points 30 s, 60 s, 2.5 min, 5 min, and then every 5 min until 30 min.

*Caffeine administration*

Caffeine was administered at the completion of the fatigue protocol. This was achieved on the heart side of the perfusion pump (arterial line) in accordance with previously published hindlimb muscle fatigue models (Howlett et al. 2005). In summary, three doses of caffeine (2.5, 5.0, 10mM in 200µl saline room temperature) were presented in a balanced order, with five minute intervals between each dose to allow complete washout of the hindlimb. Hypoxia was maintained throughout and twitch contractions (2Hz, 7-12V, 0.05ms) were monitored during each caffeine dose.

*Measurements*

*Blood pressure and twitch force*

Data was referenced to ground and amplified (Onspot Australia, NSW, Australia). The data acquisition software (Labview for Windows, National Instruments, Austin, TX ) was used to collect pressure and twitch force (data sampling rate: 200Hz).

The researcher carrying out the experimental procedure and data analysis was blinded to the specific diet group. Twitch characteristics were recorded continuously and analysed at times corresponding to the blood sampling. Developed tension and other characteristics were averaged from ten consecutive contractions. Peak tension was defined as the highest developed force in each twitch curve. Maximal peak twitch tension was defined as the mean tension over the 10 highest peak tensions. The first derivative of developed tension was used to determine the maximum rate of developed tension and relaxation. The area under the

227 twitch curve (tension-time index) was calculated relative to peak tension at the point of
228 initial (first 10 contractions), maximum (maximal 10 contractions) and after 30 min of
229 contraction (final 10 contractions). The efficiency index was calculated as the relative force
230 produced per unit of oxygen uptake by the active skeletal muscle mass.
231
232 *Blood samples*
233 Arterial and venous blood samples were collected via re-sealing silicone sections of the
234 cannulae proximal and distal to the hindlimb. Of the 200μL blood sampled, 80μl was
235 presented to the laboratory blood gas and electrolyte machine (ABL77, Radiometer,
236 Copenhagen) for measurement of $PO_2$, $PCO_2$, electrolytes, pH and haemoglobin. The
237 remaining 120 μL venous sample was spun down on a bench top micro centrifuge (Milipore,
238 NSW, Australia), the plasma removed and frozen for later analysis of lactate. Erythrocytes
239 from the plasma collections were re-suspended in an equal volume of normal saline and re-
240 injected into the venous side of the perfusion. Haemoglobin levels were maintained above
241 12.5 $g.100mL^{-1}$ whole blood throughout.
242
243 *Plasma lactate*
244 Stored venous plasma samples (200μl aliquot) were thawed on ice and analysed for plasma
245 lactate (Sigma Diagnostics, Australia). The method was adapted to the Cobas Mira (Roche
246 Diagnostics, Australia) where absorbance was read at 340nm wavelength and compared to
247 prepared controls (Sigma Diagnostics, Australia).
248
249 *Muscle samples*

At the completion of each experiment, while the blood was still flowing, the primary muscles of contraction (gastrocnemius, plantaris, soleus) were sampled, weighed, freeze-clamped and stored (-80ºC) for analysis of muscle glycogen. In addition, all muscles of the lower hindlimb were separated and weighed to account for perfused tissue mass. The right, non-perfused leg was used as a within animal control, whereby the gastrocnemius, plantaris, soleus were sampled immediately after the right femoral artery ligation in preparation for its cannulation.

*Muscle glycogen*

The muscle samples (100mg) were placed into 2ml potassium hydroxide (1M) and heated (30minutes, 70˚C water bath). Upon cooling, 100µl aliquots in duplicate, along with a standard (0-100µl) were dropped onto Whatman 3mm chromotography paper (Sigma Diagnostics, Australia). When dry, samples were washed with 2 x 2ml ethanol to remove free glucose. The remaining glycogen was set with 1ml acetate. Amyloglucosidase and 2ml glucose assay mixture (Roche Diagnostics, Australia) were added to each duplicate sample. The colour-metric reaction (45minutes, room temperature) was stopped by removal of 200µl and read at A510nm (Apollo 11- micro-plate reader, Berthold, Australia).

*Statistical Analysis*

Two-way ANOVA was conducted with diet and time main effects and diet × time interaction in a repeated measures design using Statistix, Version 8 (Analytical Software, Tallahassee, FL). A paired t-test was conducted separately to first compare peak contractions during normoxia and hypoxia induction. Individual dietary pairwise comparisons were conducted using a corrected Bonferronni post hoc analysis for multiple comparisons of individual means. Type I error, $\alpha$ was set at $P<0.05$. Data is expressed as mean ± SEM.

275

276

277

278 **Results**

279 At the time of surgery, the body mass of the n-6 PUFA (419±13g) and FO (466±53g) groups
280 was greater than the SF (370±22g) ($P<0.05$). However, this was not reflected in the total
281 lower hindlimb (SF: 5.42±0.27; n-6 PUFA: 5.34±0.14; FO: 4.95±0.13 g) or gastrocnemius-
282 plantaris-soleus (SF: 3.04±0.09; n-6 PUFA: 3.10±0.10; FO: 2.70±0.10 g) muscle mass where
283 the FO group had a small, although statistically significant, difference in mass (lower) for
284 both sets of combined tissues compared to the SF and n-6 PUFA ($P<0.05$).

285

286 *Effects of hypoxia in muscle at rest*

287 During normoxic conditions, the hindlimb demonstrated vasoactive tone (perfusion pressure:
288 SF: 117±10; n-6 PUFA: 133±5; FO: 127±9 mmHg). Oxygen saturation of the red blood cells
289 (SF: 98.2±0.2; n-6 PUFA: 98.3±0.1; FO: 98.6±0.2%) and corresponding high arterial oxygen
290 content (SF: 19.5±0.8; n-6 PUFA: 19.5±0.6; FO: 19.2±0.5 $ml.100ml^{-1}$) was achieved in all
291 dietary groups. Arterio-venous oxygen difference (SF: 3.49±0.44; n-6 PUFA: 3.67±0.60; FO:
292 3.56±0.66 $ml.100ml^{-1}$) demonstrated resting oxygen uptake physiologically typical of non-
293 stimulated normoxic conditions (SF: 0.28±0.05; n-6 PUFA: 0.29±0.06; FO: 0.31±0.06
294 $\mu mol.g.min^{-1}$) where were no differences between dietary groups ($P>0.05$). During hypoxic
295 ventilation the $P_aO_2$ [~65-70mmHg], arterial oxygen saturation (SF: 90.0±1.5; n-6 PUFA:
296 89.0±2.1; FO: 88.9±1.3 % $P<0.05$ v normoxia) and arterial oxygen content (SF: 16.7±0.5; n-6
297 PUFA: 16.4±0.5; FO: 17.0±0.5 $ml.100ml^{-1}$ $P<0.05$ v normoxia) fell in all dietary groups.
298 Nonetheless, hypoxia was not associated with changes in either the arterio-venous oxygen

299 difference (SF: 3.98±0.45; n-6 PUFA: 3.70±0.81; FO: 3.96±0.90 ml.100ml$^{-1}$) or resting
300 oxygen consumption (SF: 0.31±0.04; n-6 PUFA: 0.29±0.06; FO: 0.33±0.08 μmol.g.min$^{-1}$)
301 compared to normoxia (P>0.05). Haemoglobin concentrations were maintained high
302 throughout the procedure and were not altered by either diet or conditions (Normoxia SF:
303 13.4±0.5; n-6 PUFA: 13.3±0.3; FO:13.3±0.8 grams.100ml$^{-1}$and Hypoxia SF: 13.5±0.6; n-6
304 PUFA: 13.3±0.3; FO: 13.3±0.2 grams.100ml$^{-1}$).
305
306
307 *Effects of hypoxia during muscle contraction*
308 During normoxic conditions FO skeletal muscles developed significantly higher maximum
309 peak force than SF group (Figure 1). During hypoxia, stimulated twitch tension increased
310 over the first 60-80 s (staircase effect) to reach maximum tension, plateaued, and then
311 declined over time until end stimulation at 30 min (P<0.05) in all dietary groups. The
312 maximum peak twitch tension developed in the first 60 s of stimulated contraction was
313 significantly reduced during hypoxia relative to normoxia in all dietary groups (SF: 76%; n-6
314 PUFA: 71%; FO: 73%) (Figure 1). However the absolute developed twitch tension was
315 always greater in the FO skeletal muscle compared to either n-6 PUFA or SF groups for the
316 first 15 min (P<0.05). In addition, the greater developed tension in the FO group was
317 associated with a significantly longer time to fall to 50% of maximal twitch tension compared
318 to the SF and n-6 PUFA groups (p<0.05) (Figure 1). There was no change in resting tension
319 during the 30 min of stimulation for any dietary group. The maximum rate of force
320 development remained significantly greater in the FO skeletal muscles compared to both n-6
321 PUFA and SF for the first 20 min (Figure 2).
322
323 Oxygen consumption increased significantly (P<0.05) from the commencement of stimulated
324 muscle contractions in all groups (Figure 3), reaching steady state after 5-10 min before

declining. Oxygen consumption increased more in the FO group, reaching a higher steady state ($P<0.05$) that was achieved later than in the n-6 PUFA and the SF groups. Efficiency index (Figure 3) declined significantly in all groups over the 30 min of contractions as contractile force declined. Both FO and n-6 PUFA groups maintained a significantly higher efficiency index than the SF group over the 30 min contractions.

Venous blood lactate concentration (at rest SF: 4.85±0.29 n-6 PUFA: 5.20±0.77 FO: 5.50±0.80 $mmol.L^{-1}$) and pH (at rest SF: 7.37±0.01 n-6 PUFA: 7.27±0.04. FO: 7.31±0.02) from the perfused muscle were significantly altered during twitch contractions in all diets ($P<0.05$). By 30 min of contractions, the FO group had significantly higher hindlimb venous lactate concentrations (SF: 8.11±0.72 n-6 PUFA: 10.80±0.97 FO: 14.94±1.20 $mmol.L^{-1}$ $P<0.05$) and lower hindlimb blood pH (SF: 7.12±0.04 n-6 PUFA: 7.18±0.03 FO: 7.05±0.08 $P<0.05$) compared to the SF and n-6 PUFA groups.

*Caffeine stimulated recovery*

Established fatigue was equal with respect to absolute developed tension in all dietary groups at the end of 30 min of contractions (Figure 4). Bolus perfusion with caffeine produced dose-related recovery of developed tension (caffeine effect; $P<0.01$). Caffeine-induced contractile recovery was greater in FO animals at both 5mM and 10mM compared to the SF and n-6 PUFA animals (10nm caffeine: SF; 41±3, n-6 PUFA; 40±4, FO; 52±7 % of initial unfatigued peak force; $P<0.05$ FO versus SF, n-6 PUFA) (Figure 4). This was supported by a significantly higher maximum rate of tension development after 10mM caffeine in the FO group (SF: 3899±714 n-6 PUFA: 3326±67 FO: 5471±954 $g.s^{-1}$, $P<0.05$)

*Muscle glycogen*

Muscle glycogen concentration was significantly less in the stimulated hindlimb compare to the control (Figure 5). There was significantly less depletion of glycogen in the soleus compared to the plantaris and the gastrocnemius muscles. There was no effect of diet on muscle glycogen concentration in either the control or stimulated muscles (Figure 5).

**Discussion**

This study has confirmed that an acute bout of hypoxia reduces maximal twitch tension development and maintenance of repeated peak twitch tension, in agreement with others (Stainsby *et al.* 1990). Nonetheless, the rate of decline in muscle contraction was attenuated by dietary DHA-rich fish oil, compared to either saturated fat or n-6 PUFA diets. Furthermore, the fish oil protected against several characteristics of low frequency muscle fatigue, most notably by enhancing the contractile force recovery induced by caffeine, which in turn implies improved calcium handling ability of the skeletal muscle cells (Allen *et al.* 2008). Therefore, provision of dietary DHA-rich fish oil, known to modulate membrane composition (Peoples and McLennan 2010), in particular DHA in muscles susceptible to fatigue (Henry, *et al.* 2015), is a non-haematological approach to improve skeletal muscle contractile performance during acute low oxygen exposure and supports our hypothesis.

Varying the dietary fatty acids had no effect on resting oxygen consumption during normoxia or hypoxia. As such, the basal demand of the skeletal muscle to maintain ATP production through oxidative phosphorylation was not challenged under these conditions. In heart, the same observations are apparent, where the basal myocardial oxygen consumption following $K^+$ arrest, does not differ with dietary fish oil (Pepe and McLennan, 2002). Previous examples, using hindlimb skeletal muscle models exposed to only well oxygenated blood, support this concept (Peoples and McLennan 2010; Peoples and McLennan 2014).

Furthermore, the provision of LC n-3 PUFA via fish oil in the human diet has no significant effect on whole body oxygen consumption when metabolic demand is low (Gerling *et al*. 2014), only differing during exercise resulting in exhaustion (Peoples *et al.* 2008).

When the hindlimbs were stimulated to contract, the metabolic requirement was elevated, demonstrated both in the lowering of blood pH and the reduction of muscle glycogen across all groups. Nonetheless, animals fed fish oil were able to sustain greater force production and perform more contractions to the point of fatigue, despite no differences in muscle glycogen concentration in either the control of stimulated muscle between the groups. Notably, sustained work capacity of the fish oil group was also reflected by significantly higher circulating blood lactate concentration and lower pH, demonstrating neither are primarily implicated in reducing force capacity (Allen, *et al.* 2008) and are simply markers of work. When skeletal muscle is stimulated to contract, the oxygen requirement of the cell is increased (Grassi *et al.* 2000). Skeletal muscle force production is coupled to oxygen availability (Hogan *et al.* 1994) where hypoxia significantly reduces muscle power (Stainsby, *et al.* 1990) and this is clearly demonstrated in the current study. Thus there is a biological link between the oxygen availability and the individual energy demands of the muscle cell (Arthur *et al.* 1992), whereby the oxygen cost of contraction is important to maximise sustained work capacity (Jones, *et al.* 2011), and in the case of the current study, economy was higher in the fish oil compared to the saturated fat fed animals during the early stages of contraction.

Contractile economy in the blood perfused rat hindlimb is expressed through the ability of the skeletal muscle to sustain repetitive contractile force (Peoples, *et al.* 2013) reported as a reduced slow component of oxygen consumption in canine preparations (Zoladz, *et al.* 2008). This improved economy was clearly evident in the hindlimbs of animals fed DHA-rich fish

oil. The reduction in slow component was not immediately obvious, as the maintained contractile function actually required greater oxygen consumption; however it was demonstrated through improved efficiency and in agreement with the mirror effect termed by others (Zoladz *et al.* 2008). Under conditions where blood flow is allowed to adjust, as *in vivo*, oxygen delivery, independent of blood flow, is regulated to match metabolic demand (Mortensen *et al.* 2014). Improved contractile economy following fish oil diet is also observed using this latter model in rats with induced chronic heart failure. Animals fed a diet containing fish oil experience lower blood flow requirements during muscle contraction compared to controls animals (Holdsworth *et al.* 2014).

A lower oxygen cost of contraction may partly be explained by a change in mitochondrial function. In humans, fish oil supplementation was recently demonstrated to increase DHA concentration in the skeletal muscle mitochondrial membranes. As a physiological consequence, both the ADP sensitivity and reactive oxygen species emissions increased in these mitochondria with no evidence of oxidative damage (Herbst *et al.* 2014). Thus, when membrane DHA concentrations are optimised in skeletal muscle, including the mitochondria, there is a similar improvement in oxygen efficiency as reported in metabolically active heart (Pepe and McLennan 2002; Abdukeyum, *et al.* 2008) and protection against cellular damage in low oxygen ischemic scenarios.

Caffeine administration in hindlimb perfusion models, including the canine, partly reverses contractile fatigue and suggests improved calcium release (Howlett et al. 2005). Our results demonstrated, dietary DHA-rich fish oil also attenuated the loss of the maximum rate of developed tension and enhanced recovery of tension in response to the presentation caffeine. Specifically, caffeine facilitates neuromuscular function at the level of the sarcoplasmic

reticulum to induced $Ca^{2+}$ release (Weber *et al.* 1968; Freyer *et al.* 1989; Block *et al.* 1992) and reverse the decline in force production (Edwards 1981). The hindlimbs from the animals fed DHA-rich fish oil enhanced recovery with the caffeine and therefore suggests DHA also promotes sarcoplasmic reticulum $Ca^{2+}$ handling in skeletal muscle as previously demonstrated in the beating heart during reperfusion (Taffet *et al.* 1993; Pepe *et al.* 1999; Pepe and McLennan 2002). In skeletal muscle, hypoxia also exacerbates these conditions (Brotto, *et al.* 2000) where reactive oxygen species and free radicals are elevated (Duranteau *et al.* 1998). In fact, faced with limiting 'metabolic catastrophe' (MacIntosh, *et al.* 2012), incorporation of DHA into skeletal muscle membranes may be acting to pre-condition the cell for low oxygen scenarios, such as hypoxia, and thereby limit the oxygen slow component of muscle associated with fatigue (Jones, *et al.* 2011) and be expressed as sustained contractile force. This concept is also reflected in hearts subjected to ischemia-reperfusion whereby mitochondrial superoxide dismutase antioxidant activity of mitochondria is upregulated by prior incorporation of LC n-3 PUFA in animals fed a diet containing fish oil (Abdukeyum *et al.* 2016).

We have demonstrated that providing DHA in the diet supports skeletal muscle contractile responses during acute hypoxia. Nonetheless, collectively studies of comparative physiology also associate increased muscle membrane DHA to improved exercise performance, either as environmental adaptation or from diet and support the nutritional pre-conditioning role of incorporated membrane DHA. Chronic exposure to hypoxia (simulated altitude) has itself been shown to stimulate increase membrane content of DHA in excitable cells of rats (Jezková *et al.* 2002) independent of diet and supports the notion of oxygen management. Equally, oxygen management is also reported in migrating birds exposed to altitude through PUFA modification (McWilliams *et al.* 2004) and seals required to perform muscle

452 contractions during extended breath hold diving (Kanatous *et al.* 1999). These cases highlight
453 the combined relationship of the adaption of membrane fatty acid composition to exercise
454 performance during hypoxic challenges.
455
456 Other nutritional approaches, such as dietary nitrate (Clements *et al.* 2014) and sodium
457 bicarbonate (Berger *et al.* 2006) also reduce the slow component of oxygen consumption and
458 exercise tolerance on the basis of oxygen delivery and metabolic stability respectively. The
459 current study supports the premise that dietary fish oil may be a novel nutritional approach to
460 reduce the energy cost of exercise and, through nutritional pre-conditioning, it may support
461 continuing muscle contractions during exercise scenarios involving oxygen limitations.
462 Already, fish oil supplementation has been demonstrated to improve whole body exercise
463 economy in trained cyclists (Peoples *et al.* 2008), equivalent to the improvements achieved
464 via short term altitude acclimatization (Katayama *et al.* 2004). Furthermore, in animals, the
465 provision of LC n-3 PUFA to high altitude rescue dogs improved their rescue times (exercise
466 performance), lowered heart rates together with lower expression of oxidative stress makers
467 (Grandjean *et al.* 1998) where acute hypoxia is known to accelerate their production
468 (Ottenheijm *et al.* 2006). Given that there are many clinical examples of poor oxygen supply
469 to skeletal muscle, such as chronic obstructive pulmonary disease, where muscle fatigue is a
470 primary symptom, the provision of dietary fish oil has many possibilities in the nutritional
471 treatment of skeletal muscle pathogenesis and improved quality of life.
472
473 In conclusion, provisions of dietary fish oil, providing DHA, delayed fatigue when exposed
474 to acute bout contractions during hypoxic stress. In addition to the modulation of oxygen
475 consumption during active contractile tension, preliminary evidence in this study suggests

476 that improved recovery is partly explained by caffeine induced $Ca^{2+}$ release and therefore
477 similar to that seen in heart during ischemic-reperfusion.
478
479
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489
490 **Competing interests**
491 The authors declare that they have no conflicts of interest.
492
493 **Author contributions**
494 Authors Dr. Gregory Peoples and Professor Peter McLennan contributed equally to all parts
495 of the study including design, analysis and manuscript preparation.
496
497 **Ethical standards**
498 Experiments were assessed and approved by the Animal Ethics Committee from the
499 University of Wollongong, and all national and institutional guidelines were followed.
500

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667

668 **Figure Legends**

669

670 **Figure 1: A:** Isometric twitch tension ($g.g^{-1}$ wet-weight) of the gastrocnemius-plantaris-
671 soleus muscle bundle over 30minutes of stimulation during arterial hypoxia. **B:** Time (s) for
672 contractile tension to decline to 80% and 50% of peak. † $P<0.05$ normoxia versus hypoxia
673 peak twitch tension. * $P<0.05$ for FO versus n-6 PUFA and SF. ** $P<0.05$ for time
674 independent of diet. a,b denotes statistical difference between groups: Values displayed as
675 Mean ± SEM.

676

677 **Figure 2:** Maximum rate of contraction ($dT/dt\ g.s^{-1}$) and relaxation ($-dT/dt\ g.s^{-1}$) of the
678 gastrocnemius-plantaris-soleus muscle bundle over 30minutes of stimulation during hypoxia.
679 * $P<0.05$ for FO versus n-6 PUFA and SF. † $P<0.05$ FO and n-6 PUFA versus SF. Values
680 displayed as Mean ± SEM.

681

682 **Figure 3: A:** Oxygen consumption ($\mu mol.g.min^{-1}$) and **B:** Efficiency Index (EI)
683 ($g.g.\mu mol.min^{-1}$) over 30minutes of stimulation during arterial hypoxemia. * $P<0.05$ SF
684 versus n-6 PUFA and FO. ** $P<0.05$ time independent of diet: Values displayed as Mean ±
685 SEM.

686

687 **Figure 4:** Twitch tension ($g.g^{-1}$ w.w) at 35minutes (established fatigue) and following 2.5,
688 5.0 and 10.0mM dose of caffeine in recovery. * $P<0.05$ for the effect of caffeine dose,
689 independent of diet. a,b denotes statistical significance where the letters are different between
690 diet for each dose of caffeine $P<0.05$. Values displayed as Mean ± SEM.

691

692 **Figure 5:** Muscle glycogen concentration ($mg.g^{-1}$w.w) in the control and stimulated
693 gastrocnemius, plantaris and soleus muscles. * $P<0.05$ stimulated (contracting) muscle versus
694 the control (no contraction), independent of diet or muscle type. a,b denotes statistical
695 significance where the letters are different within the stimulated (contracted) condition and

696 between different skeletal muscles ($P<0.05$), independent of diet. Values displayed as Mean ±
697 SEM.
698

Figure 1:

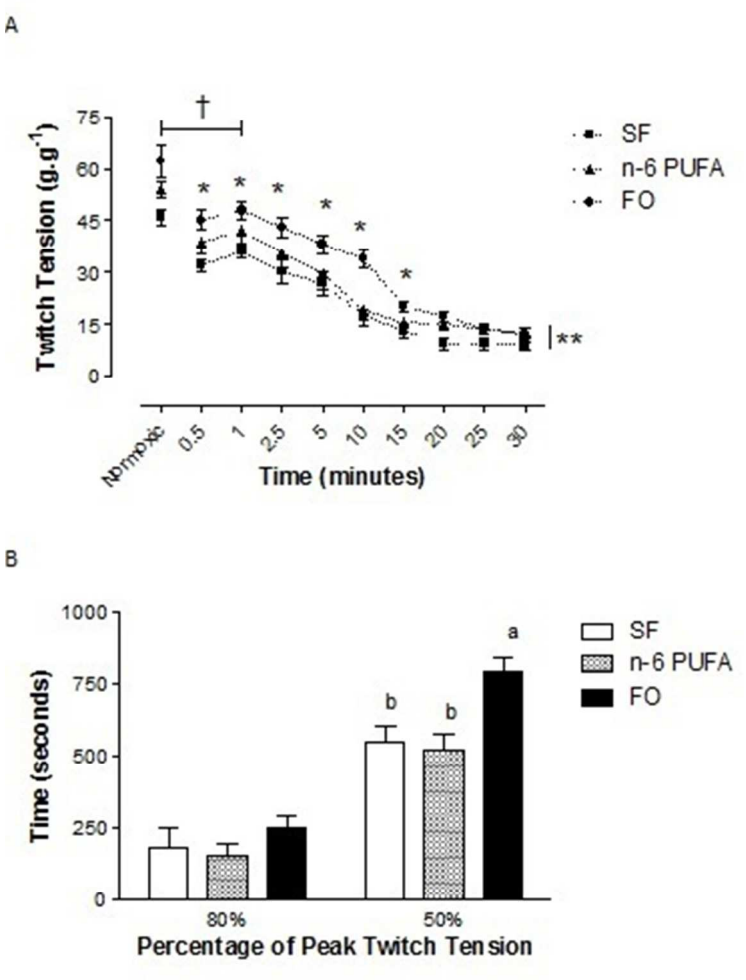


Figure 1: A: Isometric twitch tension ($g.g^{-1}$ wet-weight) of the gastrocnemius-plantaris-soleus muscle bundle over 30minutes of stimulation during arterial hypoxia. B: Time (s) for contractile tension to decline to 80% and 50% of peak. † $P<0.05$ normoxia versus hypoxia peak twitch tension. * $P<0.05$ for FO versus n-6 PUFA and SF. ** $P<0.05$ for time independent of diet. a,b denotes statistical difference between groups: Values displayed as Mean ±SEM.

Figure 1
104x152mm (96 x 96 DPI)

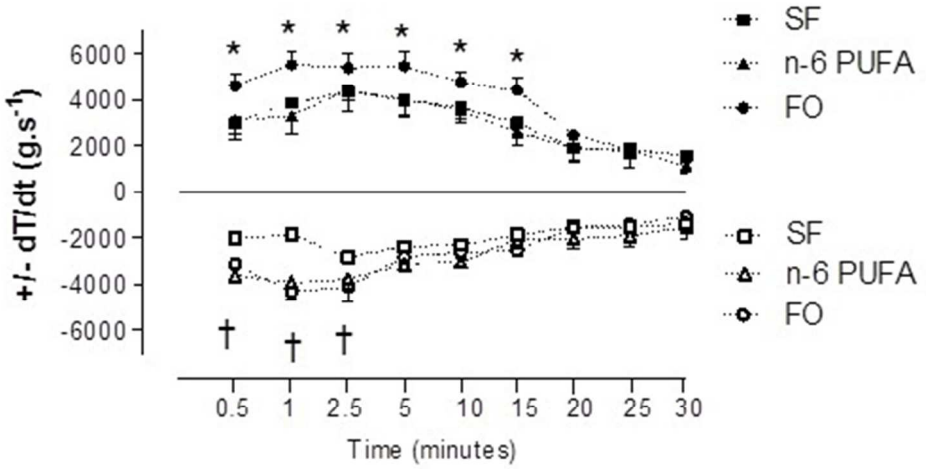


Figure 2: Maximum rate of contraction (dT/dt $g.s^{-1}$) and relaxation (-dT/dt $g.s^{-1}$) of the gastrocnemius-plantaris-soleus muscle bundle over 30minutes of stimulation during hypoxia. * P<0.05 for FO versus n-6 PUFA and SF. † P<0.05 FO and n-6 PUFA versus SF. Values displayed as Mean ± SEM.

Figure 2
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Figure 3:

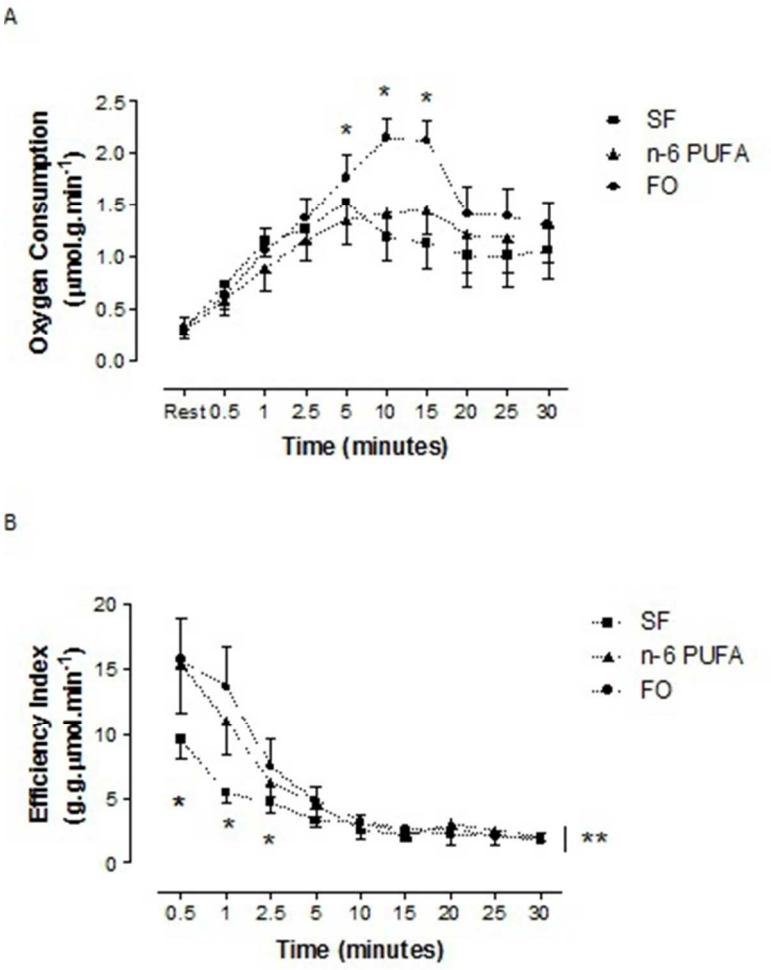


Figure 3: A: Oxygen consumption (µmol.g.min$^{-1}$) and B: Efficiency Index (EI) (g.g.µmol.min$^{-1}$) over 30minutes of stimulation during arterial hypoxemia. * P<0.05 SF versus n-6 PUFA and FO. ** P<0.05 time independent of diet: Values displayed as Mean ±SEM.

Figure 3

108x137mm (96 x 96 DPI)

Figure 4:

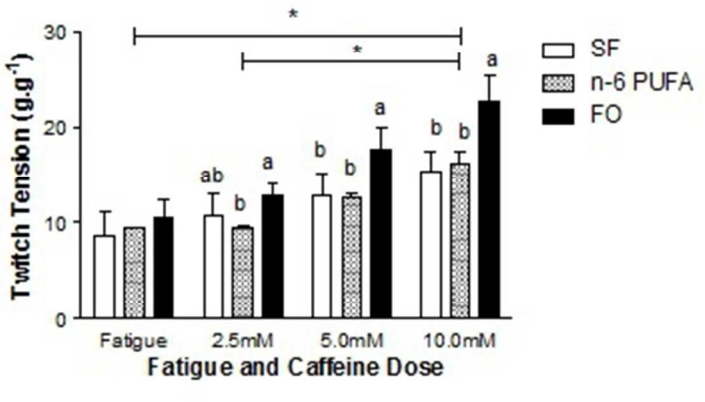


Figure 4: Twitch tension ($g.g^{-1}$ w.w) at 35minutes (established fatigue) and following 2.5, 5.0 and 10.0mM dose of caffeine in recovery. * $P<0.05$ for the effect of caffeine dose, independent of diet. a,b denotes statistical significance where the letters are different between diet for each dose of caffeine $P<0.05$. Values displayed as Mean ±SEM.

Figure 4
108x66mm (96 x 96 DPI)

Figure 5:

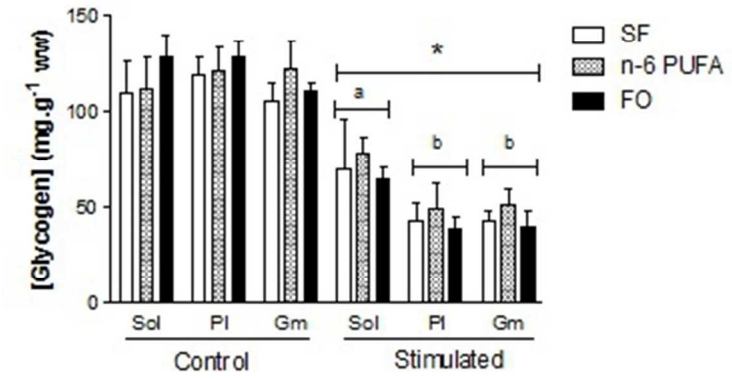


Figure 5: Muscle glycogen concentration ($mg.g^{-1}w.w$) in the control and stimulated gastrocnemius, plantaris and soleus muscles. * $P<0.05$ stimulated (contracting) muscle versus the control (no contraction), independent of diet or muscle type. a,b denotes statistical significance where the letters are different within the stimulated (contracted) condition and between different skeletal muscles ($P<0.05$), independent of diet. Values displayed as Mean ±SEM.

Figure 5
108x62mm (96 x 96 DPI)